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PRINCIPAL INVESTIGATOR: Robert E. Burke, M.D.

CONTRACTING ORGANIZATION: Columbia University
New York, NY 10032-3725

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Robert E. Burke, M.D.

E-Mail: rb43@columbia.edu

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14. ABSTRACT

A longstanding concept in neuroscience has been that the mature mammalian brain is incapable of axon regeneration. However, we have shown that it is possible to achieve long range axon growth by re-activation of intrinsic genetic programs that are active during development to mediate axon growth. We have found that activation of Akt/mTor signaling by AAV-mediated transduction with constitutively active forms of either the kinase Akt or the GTPase Rheb in a model of retrograde axonal degeneration induces axon growth by dopamine neurons. However, these molecules cannot be directly used in human therapeutics because they are oncogenes. The goal of this proposal is to develop a strategy to circumvent this problem. We have hypothesized that mediators downstream of mTor may diverge in their effects, making it possible to achieve axon growth without oncogenic risk. In Year 01 we have assessed the ability of the mTor target p70S6K to induce new axon growth. Transduction of dopamine neurons of the substantia nigra by AAV with a constitutively active form, p70S6K(delC/T389E), at three weeks after axonal destruction with the neurotoxin 6OHDA, induces new axon growth that reaches the target striatum. This growth was demonstrated by three techniques: immunostaining for tyrosine hydroxylase-positive axons in the medial forebrain bundle (MFB); quantification by confocal optical dissection of either GFP-positive axons in the MFB in transgenic TH-GFP mice, or of Tomato-positive axons following transduction with anterograde tracer Tomato-Tau.. We also determined that axons establish functional contact, demonstrated by reversal of a toxin-induced behavioral deficit. We conclude that p70S6K(delC/T389E) is an effective mediator of new axon growth.

15. SUBJECT TERMS
Axon regeneration, mTor, Akt, substantia nigra, gene therapy

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INTRODUCTION

A longstanding concept in neuroscience has been that the mature mammalian central nervous system (CNS), unlike the peripheral nervous system (PNS), is incapable of axon regeneration. There are currently two principal concepts that form the basis of our understanding of the inability of the mature brain to regenerate axons. The first, and predominant, concept is that following injury to the CNS, extrinsic factors prevent axon growth (Benowitz and Yin, 2007; Raivich and Makwana, 2007). These extrinsic factors are principally of two types: glial scar and myelin breakdown products. The second concept to account for axon regeneration failure is that as the brain matures, the intrinsic developmental genetic programs that mediate axon growth are silenced. In recent years there has been growing interest in the role played by silencing in the adult brain of these intrinsic programs. The therapeutic promise offered by the concept of silencing of axon growth programs is that it suggests that if we are able to re-activate them, it may be possible to achieve long range restorative axon growth. Such a possibility has received support from our recent studies of the activation of Akt/mTor signaling in models of retrograde axonal degeneration induced by the dopaminergic neurotoxin 6-hydroxydpamine (6OHDA). In this model, 6OHDA is injected unilaterally into the striatum of mice and within one week it induces over 80% destruction of the dopaminergic nigro-striatal projection (Ries et al., 2008). This model has been used for many years to simulate the principal neurodegeneration that occurs in human Parkinson's disease (PD). In order to accurately simulate the clinical presentation of the disease, we waited until three weeks after the lesion, when most axons have been destroyed and about 50% of neurons still survive, and then transduced the surviving dopamine neurons by use of an AAV1 vector with either a constitutively active mutant of the Akt kinase (myristoylated-Akt (MYR-Akt)) (Ries et al., 2006) or hRheb(S16H), a constitutively active mutant of the Rheb GTPase (Kim et al., 2011; Kim et al., 2012). Rheb GTPase is activated by Akt and it is a direct activator of the mTor kinase (Zoncu et al., 2011) (FIGURE 1). We have observed that transduction of SN dopamine neurons with either AAV MYR-Akt or AAV hRheb(S16H) induces a remarkable reinnervation of the striatum (FIGURE 2) (Kim et al., 2011; Kim et al., 2012). While these observations with AAV MYR-Akt and AAV hRheb(S16H) offer a promising proof-of-concept, they cannot be directly implemented as a gene therapy for humans because both of these molecules are potent oncogenes. The challenge, therefore, is to try to exploit the beneficial axon growth phenotype offered by this approach while eliminating the undesirable oncogenic phenotype. In this proposal, we seek to address this challenge by attempting to identify the critical mediators of the axon growth phenotype downstream to mTor. We

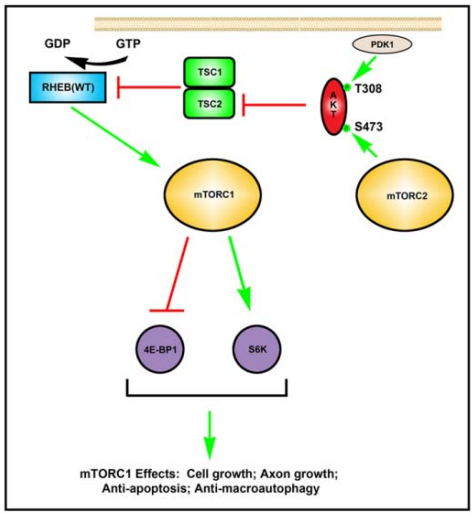


FIGURE 1. Schematic representation of Akt/Rheb/mTor signaling pathways. Following activation at the plasma membrane by phosphorylation by PDK1 and mTORC2, Akt phosphorylates and thereby inhibits the GTPase activity of the tuberous sclerosis complex (TSC). This inhibition allows accumulation of activated GTP-bound Rheb, which is a principal activator of the mTORC1 kinase. Two principal downstream substrates of mTORC1 are 4E-BP1 and p70S6K. Their phosphorylation mediates effects of mTORC1 activation.

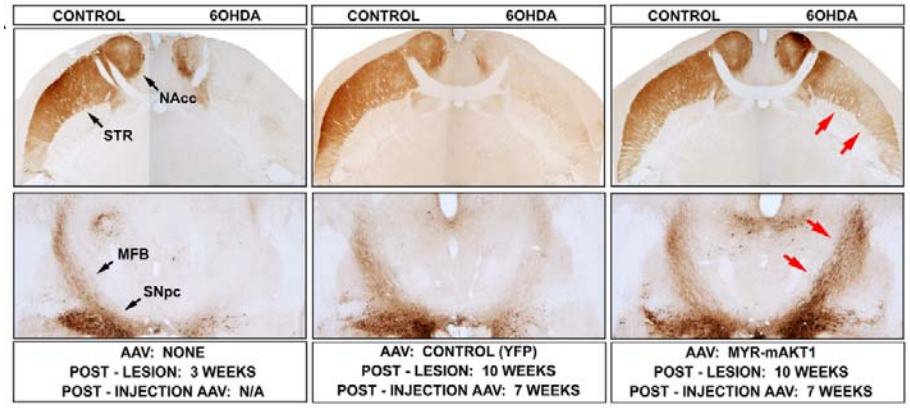


FIGURE 2. Constitutively active Akt (Myr-Akt) induces re-population of the MFB by dopaminergic axons. Horizontal sections of mouse brain, stained for TH, demonstrate restoration of dopaminergic axons within the MFB by Myr-Akt. At 3 weeks post-lesion, there are a few remaining dopaminergic neurons in the SNpc, but in this low magnification micrograph there is no remaining axonal immunostaining in the MFB or striatum (STR). Following intra-nigral administration of AAVs, very little TH immunostaining is observed in both MFB and striatum at 10 weeks post-lesion of mice given AAV YFP as control. However, robust axonal immunostaining is observed in the MFB (red arrows, bottom right panel) and in the striatum (red arrows, top right panel) of mice given AAV Myr-Akt.

postulate that at some point downstream, the paths mediating the two phenotypes must diverge. Based on our results with hRheb(S16H) we know that activation of mTor is sufficient for axon growth. mTor has two principal substrates: eukaryotic translation initiation factor 4E binding protein (4E-BP1) and p70S6K (FIGURE 1). Of the two, we hypothesize that p70S6K is more likely to play a role in axon growth. P70S6K has been identified as both necessary and sufficient for axon specification in primary neurons (Morita and Sobue, 2009). Transduction of neurons with a constitutively active form of p70S6K induced the formation of multiple axons, whereas increased expression of eIF-4E did not (Morita and Sobue, 2009) . Although our principal hypothesis favors p70S6K in the induction of axon growth, we intend in this proposal to cover all possibilities by investigating the alternate mTor mediator, 4E-BP1, as well. The aim of this proposal therefore is to test the hypothesis that p70S6K or 4E-BP1 has the ability to induce axon regeneration in lesioned dopamine neurons. This proposal has direct therapeutic implication for the treatment of PD and other chronic neurologic diseases characterized by axon degeneration.

BODY

TASK 1 YEAR 01: To determine whether p70S6K or a constitutively active mutant, or both, are mediators of axon growth in the dopaminergic nigro-striatal projection.

In Year 01 we have successfully completed this Task, and have found that p70S6K can mediate new axon growth. In order to proceed, we first created the AAV vectors for p70S6K(WT) and the constitutively active form p70S6K(del C/T389E) (Yan et al., 2006; Sato et al., 2008). This was done on schedule. We next needed to determine whether these vectors were capable of successful transduction of dopamine neurons of the substantia nigra. This was done, and both vectors were found to have excellent transduction efficiency (FIGURE 3). Before proceeding with the experimental studies in lesioned mice, as proposed within TASK 1, it was necessary to assess effects of these vectors on the dopaminergic nigro-striatal projection of normal, unlesioned mice, as a baseline, to make sure that there were no toxic effects, or, conversely, to see if they may have axon growth effects even in the absence of a lesion. This was done, and p70S6K(del C/T389E) was found to have no toxicity; on the contrary, it modestly increased the

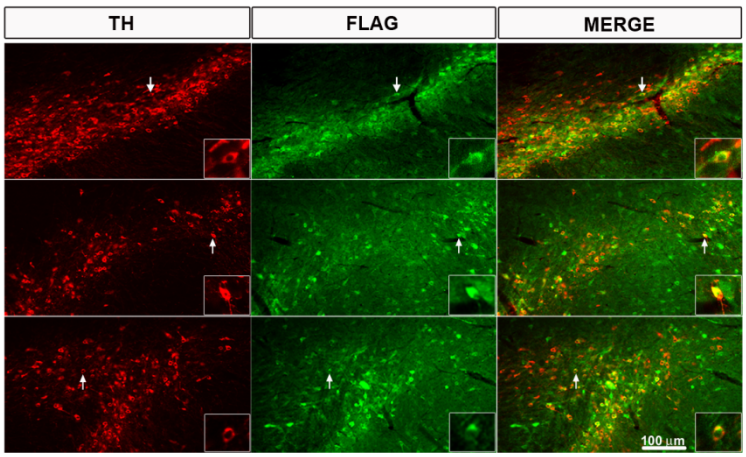


FIGURE 3. Representative epifluorescence images from three different rostro-caudal planes of the SN confirm the expression of constitutively active p70S6K (FLAG: green) within dopamine neurons of the SNpc, identified by tyrosine hydroxylase (TH) staining, following transduction with AAV. It can be seen that in all planes good efficiency of transduction was obtained. Scale bar = 100µm.

dopaminergic innervation of the striatum (FIGURE 4). p70S6K(WT) also showed no toxicity, but unlike p70S6K(del C/T389E) it had no effect on striatal dopaminergic innervation.

After the viral vectors were created and characterized, we turned our attention to the performance of the Experiments outlined in TASK 1. In each of these Experiments a 6OHDA lesion was performed, followed in three weeks by intra-nigral injection of the AAV vectors. The effects on axon growth were assessed at 12 weeks post AAV, to allow enough time to fully assess the effects of the AAV injections. We determined by several measures that p70S6K(del C/T389E) induces new axon growth following their destruction by neurotoxin. We first determined that p70S6K(del C/T389E) partially restores axon numbers in the medial forebrain bundle (MFB), identified as TH-positive axons in the MFB (FIGURE 5). p70S6K(WT) did not have an ability to induce axon growth.

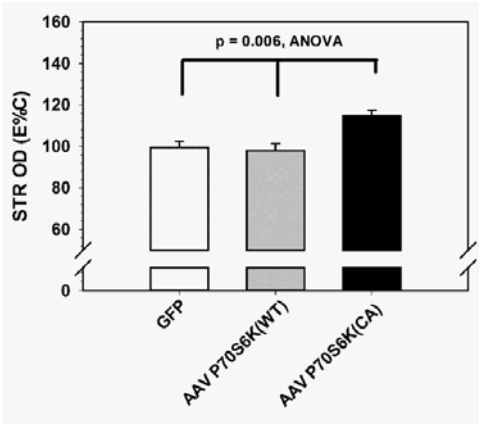


FIGURE 4. Normal adult male mice were injected with constitutively active AAV p70S6K(delC/T389E) and sacrificed at 4 weeks for histological analysis. These mice showed a modest (15%) increase in the optical density of TH immunoperoxidase staining.

Examination of TH immunostaining alone is not an adequate assessment of new axon growth in this model, because during new axon growth, the new, immature axons may not express the TH protein, and they would be overlooked. To address this concern, we used two methods. In the first, we used transgenic mice (TH-GFP) that express GFP under the TH promoter. Under the TH promoter, GFP is exclusively expressed in catecholaminergic neurons. GFP expression is more robust and more readily detected that endogenous TH, which must be demonstrated by immunostaining. Second we have used an anterograde tracer technique in which the axon-targeted fusion protein Tomato-Tau is delivered to SN neurons by AAV and expression is driven by the robust chicken-beta actin promoter, which is less likely to be subject to developmental regulation. There two methods can be performed simultaneously in the same mouse by injection of TH-GFP mice with AAV Tomato-Tau. FIGURE 6 shows that when GFP-positive axons in the MFB are quantified by optical dissection using confocal microscopy, there is a clear increase in axon number following AAV p70S6K(del C/T389E). In the same mice, Tomato-labeled, red fluorescent axons were determined, and by this assessment as well, there was a clear induction of axon growth by AAV p70S6K(del

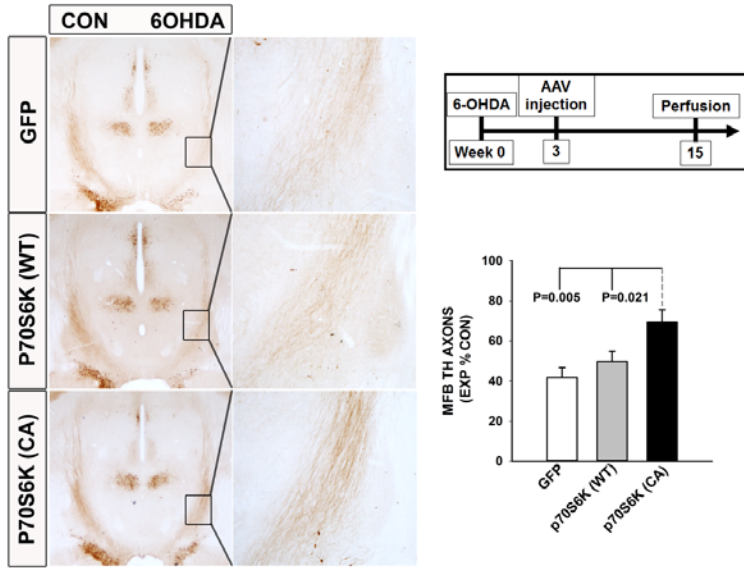


FIGURE 5. Mice treated with p70S6K(delC/T389E) showed a significant increase in the number of TH positive axons in the MFB as assessed by stereology (n=9) when compared to GFP (n=8, 28% increase) or p70S6K (WT) (n=8, 20% increase) injected mice.

C/T389E) (FIGURE 7). Thus, three different methods indicated a clear induction of new axon growth in AAV p70S6K(del C/T389E) and we conclude that our prediction is confirmed, p70S6K(del C/T389E) is capable of recapitulating the axon growth–inducing effects of Akt and hRheb(S16H).

In order to determine whether these morphologic observations have a functional counterpart, we performed a behavioral analysis, using amphetamine-induced rotational behavior. Following unilateral 6OHDA lesion, the administration of amphetamine, which induces dopamine release, results in more release on the intact side and consequently rotations away from that side, towards the side of the lesion. In mice treated with AAV p70S6K(del C/T389E), this effect is reversed, indicating that nigro-striatal dopamine release has been restored on the lesioned side (FIGURE 8).

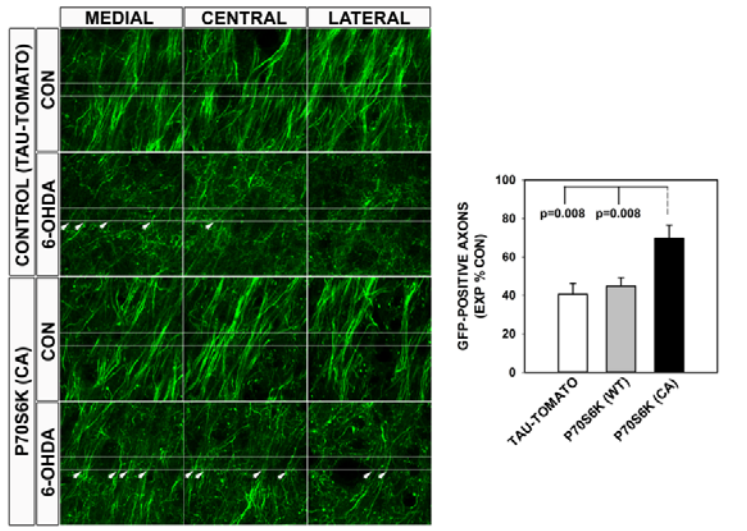
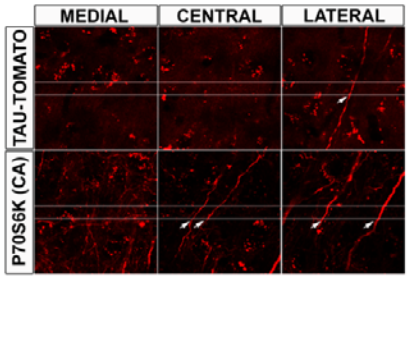


FIGURE 6. Representative confocal maximal projections of GFP fluorescence acquired from the MFB of TH-GFP mice injected with either Tau-Tomato (anterograde tracer) alone (as a control) or in combination with p70S6K(delC/T389E) three weeks following 6-OHDA injection. Quantitative analysis of GFP-positive axons reveals that mice injected with AAV-Tau-Tomato control (N = 6) had only 40% of the number of MFB axons as on the non-lesioned, contralateral control side. Mice treated with p70S6K(delC/T389E) (N = 5) had 60% of the number in the control side, indicating a restoration of 20% of the normal axon number.

Based on these results we conclude that our hypotheses is confirmed, p70S6K, a downstream target of mTORC1, is capable of recapitulating the axon growth effects of MYR-Akt and hRheb(S16H).



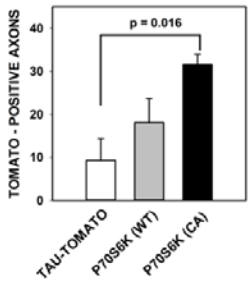
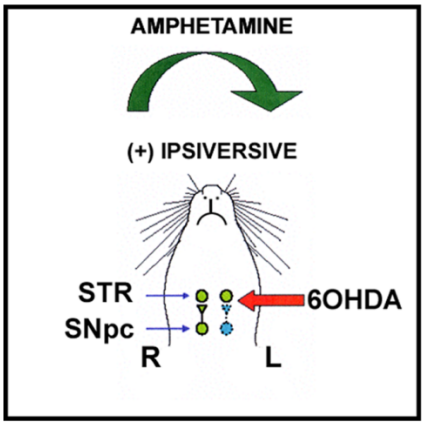


FIGURE 7. Representative confocal maximal projections of Tomato fluorescence acquired from the MFB of TH-GFP mice injected with either Tau-Tomato (anterograde tracer) alone or in combination with p70S6K(delC/T389E) three weeks following 6-OHDA injection. Quantitative analysis of Tau-Tomato-positive axons showed about 3 times the number in p70S6K(delC/T389E)-treated mice as compared to controls.



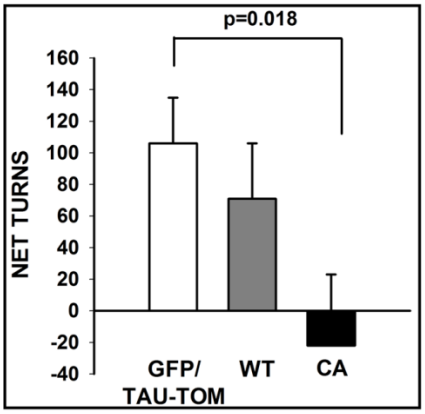


FIGURE 8. AAV p70S6K(delC/T389E)-induced axon growth reverses behavioral abnormalities induced by unilateral 6OHDA lesion. All mice underwent a test for motor recovery one week before perfusion. While p70S6K (WT) (n=11) showed no effect, a significant improvement (diminished rotational behavior) was observed with p70S6K(delC/T389E) (n=11) compared to the controls (n=13).

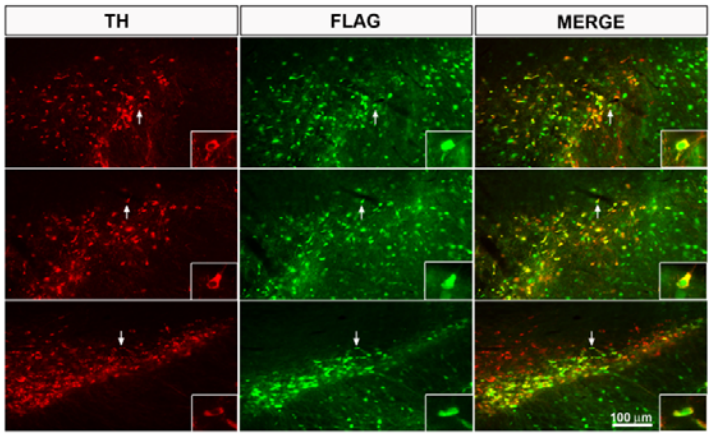


FIGURE 9. Representative epifluorescence images from three different rostro-caudal planes of the SN confirm the expression of eIF4E (FLAG: green) within dopamine neurons of the SNpc, identified by tyrosine hydroxylase (TH) staining, following transduction with AAV. It can be seen that in all planes good efficiency of transduction was obtained. Scale bar=100µm.

TASK 2. YEAR 02. To determine whether 4E-BP1, as the second principal substrate of mTORC1 signaling, is a mediator of axon growth in the nigro-striatal dopaminergic projection.

We have begun the work of TASK 2 in Year 02. We have successfully created the AAV vector for eIF4E, a protein translation factor that is constitutively inhibited by 4E-BP1. In order to mimic the inhibitory effects of mTORC1 on 4E-BP1, the appropriate strategy is to

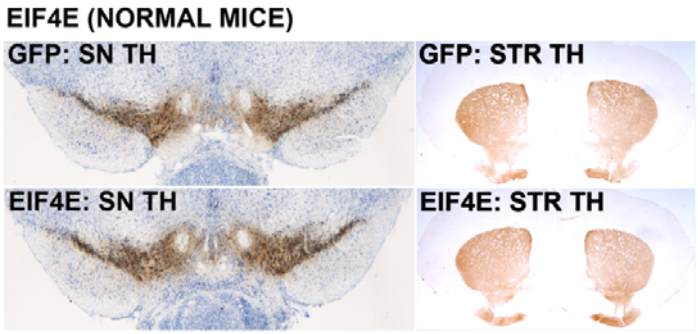


FIGURE 10. At 4 weeks following intra-nigral injection of AAV eIF4E, there is no change in the number of TH-positive neurons in the SN (left panels) or density of TH immunostaining in the striatum (right panels).

overexpress eIF4E. As we did for the p70S6K constructs, we determined whether the vector was capable of successful transduction of dopamine neurons of the substantia nigra. The vector was found to have excellent transduction efficiency (FIGURE 9). We next assessed effects of the vector on the dopaminergic nigro-striatal projection of normal, unlesioned mice, as a baseline, to make sure that there were no toxic effects, or, conversely, to see if they may have axon growth effects even in the absence of a lesion. This was done, and eIF4E was found to have no toxicity. It had no effect on dopaminergic neuron number in the SN, and, unlike p70S6K(del C/T389E), it had no effect on striatal dopaminergic innervation (FIGURE 10). With these studies now completed, we are ready to proceed with lesion experiments, as we proposed for TASK 2 in Year 02.

KEY RESEARCH ACCOMPLISHMENTS

- AAV2/1 vectors have been created for p70S6K(WT), p70S6K(del C/T389E), and eIF4E.
- All of these vectors have been shown to exhibit good transduction efficiency for dopaminergic neurons of the SN.
- AAV p70S6K(del C/T389E) has been shown to induce new axon growth in SN dopamine neurons following axon destruction, thus recapitulating the abilities of MYR-Akt and hRheb(S16H) to do so.
- The new axon growth induced by p70S6K(del C/T389E) is functional; it is able to restore behavioral deficits induced by 6OHDA lesion.

REPORTABLE OUTCOMES:

Padmanabhan S, Yarygina O, Kareva T, Kholodilov N, Burke RE. A constitutively active form of p70S6K induces axon growth in the nigrostriatal dopaminergic system. Society for Neuroscience, 2012.

CONCLUSIONS

Based on our results in Year 01, we conclude that our fundamental strategy, to attempt to recapitulate the axon growth effects of MYR-AKT and hRheb(S16H) with mediators downstream of mTORC1 is successful, because we have achieved axon growth with a constitutively active form of p70S6K. We therefore in Year 02 will continue our original plans to evaluate the axon growth capability of the second mTORC1 downstream pathway, mediated by eIF4E. In completing our studies of p70S6K, we noted that although p70S6K(del C/T389E) has clear axon growth effects, they do not seem to be as robust as

hRheb(S16H), our most potent lead molecule to date. We are therefore already beginning to develop the axon-targeting strategy proposed for TASK 3. We anticipate that axon targeting will enhance the efficacy of our lead second generation molecules without adding to their oncogenic potential.

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